AD_____

Award Number: DAMD17-03-1-0406

TITLE: The Role of AhR in Breast Cancer Development

PRINCIPAL INVESTIGATOR: Xinhai Yang, Ph.D., M.D.

CONTRACTING ORGANIZATION: Boston University
Boston, MA 02118

REPORT DATE: July 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2004	Annual Summary (1 Jul 2003 - 30 Jun 2004)

4. TITLE AND SUBTITLE
The Role of AhR in Breast Cancer Development

5. FUNDING NUMBERS
DAMD17-03-1-0406

6. AUTHOR(S)
Xinhai Yang, Ph.D., M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Boston University
Boston, MA 02118

E-Mail: xinhai@bu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

It has long been suggested that ubiquitous environmental chemicals, such as PAH, contribute to human breast cancer. Most of the biologic activity of PAH and related dioxins is mediated by the AhR. We tested if constitutively active AhR in a human breast cancer cell line regulates *c-myc*, an important breast cancer gene which contains six AhR binding sites (AhREs) in its promoter. Our results indicate that: 1) there is a significant baseline level of wildtype *c-myc* promoter driven reporter activity in these tumor cells which was not affected by inclusion of TCDD, a strong AhR agonist, 2) the baseline reporter activity was not affected by deletion of the NF-κB site, 3) while mutation of single AhRE sites had no effect on baseline reporter activity, mutation of all six sites resulted in a five fold increase in reporter activity; a similar increase in reporter activity was seen when the wildtype reporter construct was co-transfected with an AhR repressor plasmid, 4) *c-myc*-specific real time PCR indicated that AhR repressor transfection increased background levels of endogenous *c-myc* mRNA. These results suggest that the AhR represses *c-myc* transcription and that AhR up-regulation in tumor cells may represent a failed growth feedback mechanism.

14. SUBJECT TERMS
Breast cancer, AhR, *c-myc*, NF-κB, TCDD

15. NUMBER OF PAGES
22

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

DOD Training Grant Annual Summary-2004

Introduction

It has long been suggested that ubiquitous environmental chemicals, such as polycyclic aromatic hydrocarbons (PAH), contribute to human breast cancer. The preferential targeting of breast tissue by orally administered PAH in rodent breast cancer models supports this contention. Most of the biologic activity of PAH and related dioxins is mediated by the AhR. AhR activation can induce cytochrome P-450 enzymes, proto-oncogenes (e.g. *c-myc, Ha-ras, c-erb-2*) and transcription factors (e.g. NF-κB). Therefore, it is possible that AhR activation plays an important role in the initiation and progression of carcinogenesis by regulating a cascade of intracellular events involving NF-κB, *c-Myc*, and/or other proto-oncogenes. To investigate the role of the AhR in breast cancer development, we originally proposed three aims: Aim 1 is to map the contact domains between AhR and Rel A, aim 2 is to assess AhR regulation of NF-kB activity and c-myc transcription in mammary tumor cells, aim 3 is to characterize AhR regulation of cell cycle components in mammary tumor lines.

Previously, we demonstrated that the AhR and CYP1B1, an AhR-regulated gene, are dramatically up regulated in rodent and human breast tumors. In pursuit of specific aim #2, we investigated the possible consequences of this apparent constitutive AhR activation on the regulation of *c-myc*. In specific, we tested if constitutively active AhR in a human breast cancer cell line regulates *c-myc*, an important breast cancer oncogene that contains six AhR binding sites (AhREs) in its promoter.

Body

Several laboratories have considered the ability of the AhR to modulate growth through interaction with other transcription factors including NF-κB, Rb, and estrogen receptors. We evaluated the potential for the AhR to directly affect transcription of c-myc, an important oncogene which can regulate cell growth and apoptosis and which is dysregulated in many tumor types including breast cancers. AhR-mediated regulation of c-myc seemed plausible given the presence of six AREs within a 3.2 kb region of the human c-myc promoter. To evaluate the role of the AhR in regulating baseline levels of c-myc in human tumors, a luciferase reporter construct driven by the human c-myc promoter was employed. The wildtype reporter gene, reporter variants mutated in the AhR and/or NF-κB binding sites, and/or a plasmid encoding a potent AhR repressor were then transfected into a malignant human mammary tumor line, Hs578T, expressing high AhR levels, to determine the effect of a putatively active AhR on c-myc transcription.

Methods

Cell culture

The estrogen receptor negative Hs578T tumor cell line was derived from a human mammary carcinoma and is epithelial in origin. The non-transformed Hs578Bst myoepithelial cell line was derived from normal tissue adjacent to the tumor from which Hs578T cells were derived. Both lines were grown in a humidified, 5% CO_2 atmosphere at 37° C. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with glutamax-1 (L-alanyl-L-glutamine), supplemented with 10% fetal calf serum (FCS), penicillin (500 IU/ml), streptomycin (5 mg/ml), and L-glutamine (2 nM). All media components were purchased from Invitrogen .

Protein extraction and western immunoblotting

Total cell lysates were prepared from Hs578T and Hs578Bst cells by incubating washed cell pellets for 10 min in lysis buffer (50 mM $KHPO_4$, pH 7.4, 5 mM DTT) and 10 µl/ml protease inhibitor cocktail (Sigma, St. Louis, MO) on ice. Following a 10 min centrifugation, protein concentrations of total cell lysates were quantified using Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA). Nuclear and cytoplasmic fractions were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Equal amounts of protein (typically 40 µg) were boiled for 5 min in 1X SDS-PAGE sample buffer (50 mM Tris buffer, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue and 1% β-mercapthoethanol) before SDS-PAGE electrophoresis through a 6.8% polyacrylamide gel and overnight transfer onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Following transfer, membranes were blocked with 5% skim milk powder in 1X TBS plus 0.05% Tween-20 (TBST). The primary antibody was polyclonal rabbit anti-human AhR antibody (Santa Cruz, CA) and the secondary antibody was HRP-linked rabbit Ig-specific goat antibody (Pierce, Rockford, IL). Bands were detected using enhanced chemiluminescence substrate (Sigma, St. Louis, MO) and exposure to X-ray film (Fuji, Japan). Membranes were stripped with the Re-Blot Western Blot Recycling Kit (Chemicon, Temecula, CA) and re-blotted with β-actin-specific mAb (Sigma, St. Louis, MO) to confirm equal loading. Where indicated, blots were reprobed with α-tubulin (Oncogene Research, Boston, MA)- or lamin A/C (Novocastra Laboratories, United Kingdom)-specific mAbs to confirm the purity of cytoplasmic and nuclear protein extracts respectively.

Reporter plasmids and site-directed mutagenesis

The *pGL3-c-myc* reporter was constructed by cloning the HindIII (Blunted)-SacI human *c-myc* promoter fragment of *pVCAT* (kindly provide by Dr. D.L. Levens; NCI) into the KpnI (Blunted)-SacI sites of the *pGl-basic* luciferase reporter plasmid (Promega, Madison, WI). Deletion of two NF-κB binding sites in this plasmid was achieved by site-directed mutagenesis using two deoxyoligonucleotide primers: 5'-/5Phos/GAG TTA ACG GTT TTT TTC ACA ATG ACT CCC CCG GCT CGG-3' and 5'-/5Phos/ GGC TAT TCT GCC CAT TTG CCC GCC GCT GCC AGG-3'. The resulting plasmid was designated "*pGL3-NF-κB$_{KO}$*". This plasmid was used as the template for further site-directed mutagenesis of AhREs as described (Matikainen et al., 2001). Plasmids in which each individual AhRE or pairs of AhREs (i.e. AhRE 3 and 4) were mutated (*pGL3-AhRE$_1$Mut*, *pGL3-AhRE$_2$Mut*, *pGL3-AhRE$_{3,4}$Mut*, *pGL3-AhRE$_5$Mut*, and *pGL3-AhRE$_6$Mut)* were generated with the following primers: 5'-/5Phos/ CCG TGT GGG AGG AAT GGG GGT GGG ACG -3' *(pGL3-AhRE$_1$Mut)*, 5'-/5Phos/ CCC TAT CTA CAC TAA CAT CCC ATT CTC TGA ACG CGC GCC -3' *(pGL3-AhRE$_2$Mut)*, 5'-/5Phos/ GCA GCC TGG TAC GCG AAT GGA ATG GCG GTG GGC GCG C -3' *(pGL3-AhRE$_{3,4}$Mut)*, 5'-/5Phos/ GGG TTC CCA AAG CAG AGG GAA TGG GCG AAA AGA AAA AAG ATC C-3' *(pGL3-AhRE$_5$Mut)*; 5'-/5Phos/ CTG CCT TAT GAA TAT ATT CAT TCT GAC TCC CGG CCG GTC GG-3'*(pGL3-AhRE$_6$Mut)*. The positions of the mismatches are underlined. A plasmid in which all six AhREs were mutated (*pGL3-AhR$_{1-6}$Mut*) was produced by sequential mutation of each of the AhREs. All of the site-directed mutagenesis was conducted using the QuickChange multi site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA) according to the manufacturer's instructions. DNA sequencing was performed on each plasmid to verify the deletions/mutations and to confirm that no other sequence changes had occurred.

AhR-dependent expression of the *pGudLuc6.1*-firefly luciferase reporter construct (*pGudLuc*) is driven by four AhREs derived from the *CYP1A1* promoter. This construct was kindly provided by Dr. M. Denison (U.C. Davis).

Cloning and characterization of the *Fundulus heteroclitus AhRR* expression vector (*FhAhRR*) has been previously described (Karchner et al., 2002). The product of this vector efficiently suppresses mammalian AhR activity induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

Transient transfections, TCDD treatment, and luciferase assays

Hs578T cells (3 x 10^4/well) were plated in 12-well culture plates and cultured to 80% confluence. Lipofectamine 2000 transfection reagent (Invitrogen) was used according to the manufacturer's instructions to transfect cells. The renilla luciferase vector *phRL-TK* (0.05 μg) was co-transfected with firefly luciferase reporter constructs (0.1 μg *pGudLuc,* 0.5-1.0 μg wildtype *pGL3-c-Myc* or mutant constructs). Where indicated, 0.5 μg of *pcDNA-FhAhRR* or control *pcDNA3.1* (Invitrogen) was added to the transfection mixture. For each experiment, the amount of total DNA transfected was equilibrated with parental expression vectors. Cells were incubated overnight, washed twice with phosphate-buffered saline (pH 7.2), and resuspended in 75 μl RPMI prior to luciferase analysis. Luciferase activity was determined with the Dual Glo Luciferase system (Promega, Madison, WI) which allowed sequential reading of the firefly and renilla signals. Cells were lysed according to the manufacturer's directions (Promega, Madison, WI), transferred to 96-well white wall plates, and analyzed using a Reporter Luminometer (Promega, Madison, WI). The renilla signal was read after quenching the firefly output, thus allowing normalization between sample wells. The normalized firefly luciferase signal is expressed relative to the renilla signal.

TCDD was obtained from Cambridge Isotopes Laboratories (Andover, MD) at >99% purity and was maintained as a 1000x stock solution in anhydrous tissue culture grade DMSO. TCDD (1.0 nM) in DMSO or DMSO alone (final volume 0.1%) was added to cultures 2 hrs after transfections and cells were incubated for an additional 24 hrs. Cells then were washed twice with phosphate-buffered saline (pH 7.2) and resuspended in 75 μl RPMI for luciferase analysis.

Quantitative c-myc-specific real-time PCR

Hs578T cells were plated on two 10 cm cell culture dishes (10^6 cell/well) and cultured to 80% confluence. Equal amounts of *pcDNA-FhAhRR* or parental vector *pcDNA3.1* were added to separate plates and cells transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty four hrs later, adherent Hs578T cells were washed twice with PBS (pH 7.2) and harvested. Total RNA was isolated using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA). RNA samples were treated with RNase-free DNase according to the manufacturer's instructions. Total RNA was eluted from the columns with 60 μl RNase-free water and quantified by UV absorbance. First-strand cDNA was synthesized using 2 μg of each total RNA, random hexamers, and SuperscriptII reverse transcriptase according to the manufacturer's instructions (Invitrogen). Real-time PCR amplification mixtures (25 μl) contained 1 μl template cDNA, 2x

SYBR Green I Master Mix buffer (12.5 µl) (Applied Biosystems, Foster City, CA) and 300 nM of a forward and reverse primer pair. The sequences for *c-myc* amplification in real-time PCR were previously described (Latil et al., 2000) and are as follows: sense: 5'-ACC ACC AGC AGC GAC TCT GA- 3', antisense: 5'-TCC AGC AGA AGG TGA TCC AGA CT-3'. Human ribosomal RNA, amplified with previously published primer sequences (Nazarenko et al., 2002), was used for RNA normalization of the *c-myc* signal as previously described (Saez et al., 2003). The ribosomal RNA primers were as follows: sense: 5'-GAC TCA TTC GCC CTG TAA TTG GAA TGA GTC- 3' , antisense: 5'-CCA AGA TCC AAC TAC GAG CTT-3'. Reactions were run in an ABI PRISM 5700 Sequence Detector (Applied Biosystems) using the following standard cycling conditions: 10 min polymerase activation at 95° C, 40 cycles for 15 seconds at 95° C, and 60° C for 60 sec. Results for each experiment are calculated from three replicate PCR reactions. Threshold cycle (CT) values were collected at linearity. Relative mRNA expression was normalized against internal ribosomal controls. The parameter $2^{-\Delta CT}$, where ΔCT equals CT of the *c-myc* signal minus the CT of the endogenous ribosomal RNA control, was used to describe the relative levels of *c-myc* mRNA normalized to 18S rRNA.

Data analyses

Statistical analyses were performed with Statview (SAS Institute, Cary, NC) or Excel. Data from a minimum of three experiments are presented as means ± standard errors (SE). One-factor ANOVAs and a Fisher PLSD post hoc comparisons test or the Student's T test were used to determine significant differences.

Results

1. Constitutive nuclear AhR localization and binding to the c-myc promoter in Hs578T mammary tumor cells

Figure 1

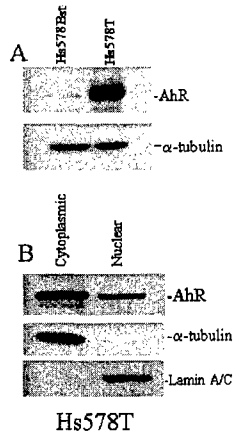

Figure 1 Hs578T human mammary tumor cells express high levels of cytoplasmic and nuclear AhR: *A) Total AhR protein was extracted from non-malignant Hs578Bst and from malignant Hs578T cells and analyzed by western blotting. Blots were stripped and re-probed for α-tubulin to confirm equal loading of wells. Representative data from a total of three experiments are presented.* ***B)*** *Cytoplasmic and nuclear cell extracts prepared from subconfluent monolayers of malignant Hs578T cells were analyzed by western immunoblotting with AhR-specific antibody following SDS-PAGE. Blots were stripped and re-probed for lamin A/C- and α-tubulin to confirm purity of the nuclear and cytoplasmic cell fractions respectively. Representative data from a total of three experiments are shown.*

A number of studies demonstrated AhR expression, usually at notably high levels, in rodent and human tumors. Furthermore, we have shown in a primary mammary tumor model that much of that AhR resides in the tumor cell nucleus, a result suggestive of constitutive AhR activation in the absence of exogenous ligands. To develop a model system in which the effects of constitutively active human AhR on gene transcription in mammary tumors can be evaluated, AhR expression, localization, and/or function were evaluated in the malignant human mammary tumor cell line, Hs578T, and in a syngeneic normal myoepithelial cell line, Hs578Bst. Non-transformed Hs578Bst cells expressed low but significant levels of AhR protein while malignant Hs578T cells expressed significantly higher levels of AhR (Figure 1A). Furthermore, approximately one third of the AhR in Hs578T cells was located in the nucleus (Figure 1B), a result consistent with constitutive AhR activation.

Figure 2

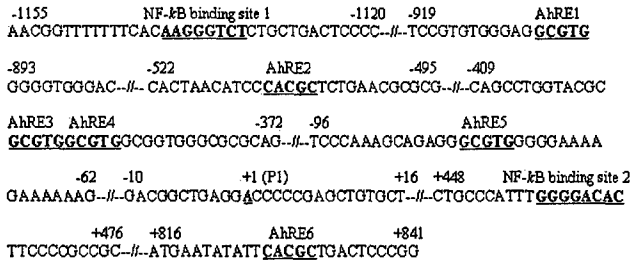

Figure 2 The human c-myc promoter contains six aryl hydrocarbon response elements (AhREs) and two NF-κB binding sites. *The locations of six consensus AhR binding motifs and two NF-κB binding sites are indicated.*

Figure 3

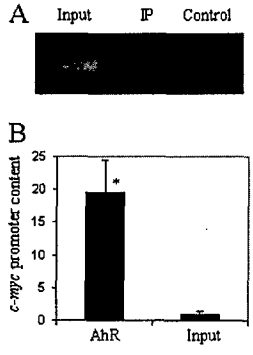

Figure 3 The AhR constitutively associates with the c-myc promoter. *Chromatin immunoprecipitation was performed on untreated Hs578T cells as described in Materials and Methods.* ***A)*** *PCR of DNA from the input fraction, AhR immunoprecipitation fraction (IP), and a no-antibody control. Representative data from a total of three ChIP experiments are presented.* ***B)*** *Recovery of the c-myc promoter from equal quantities of DNA was measured by real-time PCR and normalized to the input fraction. Data are pooled from three experiments and expressed as mean ± standard error. An asterisk (*) indicates a significant level of promoter binding relative to the input control, $p<0.02$.*

In considering possible gene targets for what appears to be active AhR in tumor cells, we noted the presence of six consensus AhR response elements (AhREs) within a 3.2 kb region of the human *c-myc* promoter (Figure 2). If the AhR affects *c-myc* gene transcription in the absence of exogenous ligands, then it would be predicted that the AhR would constitutively bind this promoter region. Chromatin immunoprecipitation experiments were performed to test this hypothesis. As seen in Figure 3A, a relatively strong *c-myc*-specific PCR signal was seen following immunoprecipitation with AhR-specific antibody and DNA amplification with *c-myc*-specific primers. Indeed, immunoprecipitation of the AhR complex resulted in almost a 20-fold increase in the *c-myc* signal relative to the input control (Figure 3B, $p<0.02$). This increase approximates the signal seen following AhR immunoprecipitation and amplification of the promoter region upstream from *CYP1A1*, a prototypic AhR responsive gene, in another human mammary tumor line.

2. AhRE-dependent regulation the of c-myc promoter

Figure 4

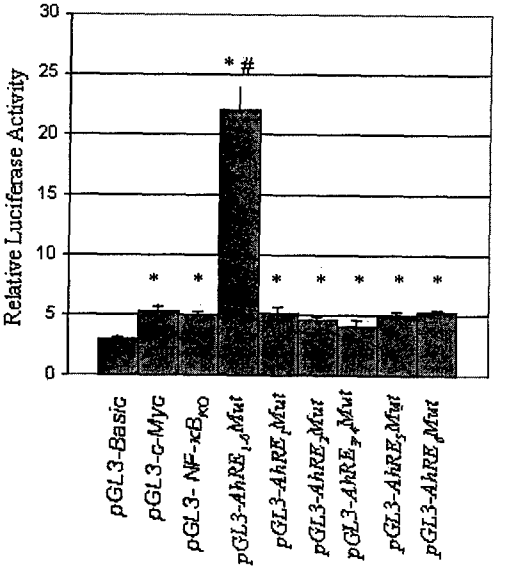

Figure 4 Deletion of all six AhREs increases constitutive c-myc promoter activity. *Hs578T cells were transfected with 0.05 μg phRL-TK and with 1.0 μg pGL3-basic, wildtype pGL3-c-Myc, a reporter construct in*

which the two NF-κB binding sites were deleted (pGL3-NF-κB$_{KO}$) or constructs in which the two NF-κB sites were deleted and individual AhREs (pGL3-AhRE$_1$Mut, pGL3-AhRE$_2$Mut, pGL3-AhRE$_5$Mut, pGL3-AhRE$_6$Mut), a pair of AhREs (pGL3-AhRE$_{3,4}$Mut) or all six AhREs (pGL3-AhRMut$_{1-6}$) were mutated. Twenty four hours later cells were harvested and lysates assayed for firefly and renilla luciferase activity. Data pooled from seven experiments are presented as the average normalized firefly luciferase activity ± standard error. An asterisk (*) indicates a significant increase in normalized reporter activity relative to background activity observed following transfection with pGL3-basic, p<0.001. A pound sign (#) indicates a significant increase in reporter activity relative to that seen in cells transfected with wildtype pGL3-c-Myc, p<0.001.

The AhR can both induce and suppress gene transcription. Therefore, a human *c-myc* promoter-reporter construct (*pGL3-c-myc*) was used to determine if the effect of constitutive AhR binding to the *c-myc* promoter effects up- or down-regulation of *c-myc* transcription. Since the 3.2 kb *c-myc* promoter region encompassing the six AhREs also contains two NF-κB binding sites, and since NF-κB is well known for its ability to regulate *c-myc*, it also was important to evaluate the possible contribution of NF-κB to background levels of *c-myc* transactivation. To address these issues, *pGL3-basic*, a control plasmid containing a minimal promoter sequence, wildtype *pGL3-c-myc*, and a series of reporter constructs mutated in the NF-κB- and/or the AhR-binding sties were transfected into Hs578T cells and reporter activity assayed 24 hrs later.

Transfection of wildtype *pGL3-c-myc* into Hs578T cells resulted in approximately a 50% increase in the background level of *c-myc* promoter activity as compared with cells transfected with the parental *pGL33basic* reporter plasmid (Figure 4A, first and second bars, p<0.001). Since mammary tumors, including Hs578T cells, frequently have elevated levels of transcriptionally active NF-κB, it was anticipated that deletion of both NF-κB-binding sites would result in a decrease in constitutive *c-myc* reporter activity. However, deletion of both NF-κB-binding sites (*pGL3-NF-κB$_{KO}$*) had no effect on reporter transactivation (Figure 4, third bar).

Mutation of individual AhREs (*pGL3-AhRE$_1$Mut, pGL3-AhRE$_2$Mut, pGL3-AhRE$_5$Mut, pGL3-AhRE$_6$Mut)* or a pair of adjacent AhREs (*pGL3-AhRE$_{3,4}$Mut*) following deletion of the two NF-κB-binding sites had no effect on constitutive *c-myc* reporter activity (Figure 4, bars 5-9). However, deletion of all six AhREs (*pGL3-AhR$_{1-6}$Mut*) resulted in a significant, 4-5 fold increase in reporter activity (fourth bar, p<0.001). These data suggest that constitutively active AhR represses baseline levels of *c-myc* transcription and that not all of the AhREs are required for AhR-mediated repression.

3. Effect of a potent environmental AhR ligand, TCDD, on AhR-mediated repression of c-myc transactivation.

A number of AhR ligands are carcinogenic. Indeed, some AhR ligands are used in animal models of mammary gland tumorigenesis. Although a principle mechanism for AhR ligand carcinogenicity is the oxidation of parent compounds into mutagenic metabolites, a process mediated by AhR-regulated CYP1 enzymes, other mechanisms, including the dysregulation of oncogenes, have been considered. Therefore, it is conceivable that binding of tumor cell AhR by exogenous environmental pollutants would release transcriptionally repressive AhR from the *c-myc* promoter thereby increasing cell growth and/or altering responses to *c-myc*-regulated apoptosis signals. Alternatively, hyper-activation of the AhR with environmental ligands could stabilize the AhR-*c-myc* complex, further inhibiting *c-myc* transactivation. To test these possibilities, the effect of a potent AhR ligand, TCDD, on AhR-mediated repression of *c-myc* reporter activity was evaluated. The effect of TCDD on a promoter known to be positively regulated by the AhR (i.e. *CYP1A1*) was evaluated first as a positive control.

Figure 5A

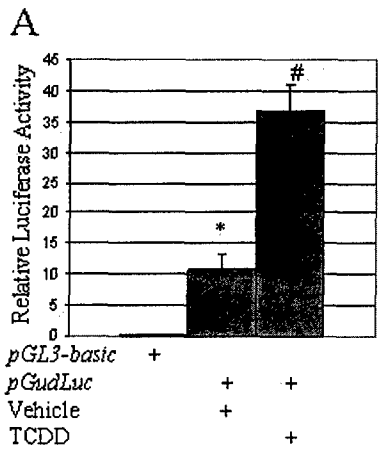

Figure 5B

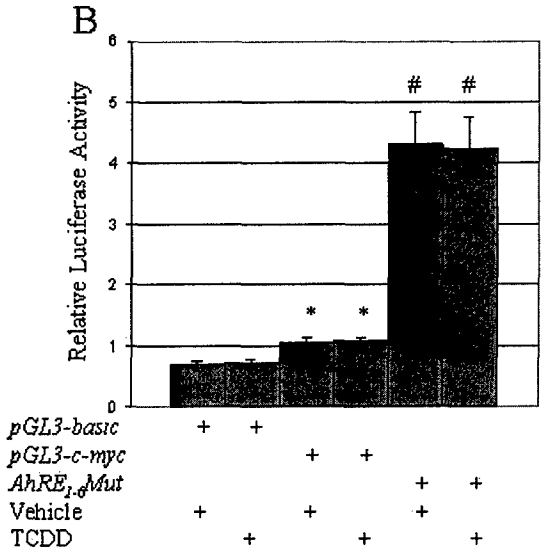

Figure 5 AhR activation with an exogenous ligand, TCDD, has no effect on c-myc reporter activity. A) *Subconfluent Hs578T cells were transfected with 0.1 μg of a control vector (pGL3-basic) or with 0.1 μg of a CYP1A1 promoter-driven firefly luciferase gene (pGudLuc) and 0.5 μg of the renilla-luciferase vector phRL-TK. Twenty four hours later cultures were treated with vehicle (0.1% acetone) or with TCDD (10^{-9} M final concentration). Cells were harvested twenty four hours later and lysates assayed for firefly and renilla luciferase activity. Data pooled from four experiments are presented as the average normalized firefly luciferase activity ± standard error. An asterisk (*) indicates a significant increase in normalized reporter activity relative to pGL-basic-transfected cells, $p<0.04$. A pound sign (#) indicates a significant increase relative to pGudLuc-transfected, vehicle-treated cells, $p<0.002$.* ***B)*** *Cells were treated as above except that 1.0 μg wildtype pGL3-c-myc or AhRE mutant pGL3-AhRE$_{1-6}$Mut was substituted for the pGudLuc reporter. Data pooled from six experiments are presented as the average normalized firefly luciferase activity ± standard error. An asterisk (*) indicates a significant increase in normalized reporter activity relative to the activity in pGL3-basic-transfected cells, $p<0.002$. A pound sign ($^{\#}$) indicates a significant increase relative to pGL3-c-myc-transfected cells, $p<0.001$.*

Transfection of a *CYP1A1* promoter-driven reporter construct (*pGudLuc*) into Hs578T cells resulted in a significant ~10-fold increase in reporter activity relative to that observed in cells transfected with the parental *pGL3-basic* plasmid (Figure 5A, second bar, $p<0.04$). Addition of 1 nM TCDD further enhanced promoter activity (third bar; $p<0.002$). This high level of constitutive and inducible reporter activity is in keeping with the presence of four AhREs in this reporter construct and the efficiency with which the AhR positively induces *CYP1A1*.

In contrast, TCDD treatment of Hs578T cells transfected with *pGL3-c-myc* had no effect on the low but significant ($p<0.002$) levels of reporter activity (Figure 5B, third and fourth bars). As in previous experiments, Hs578T cell transfection with *pGL3-c-myc$_{1-6}$Mut* significantly increased the level of constitutive reporter activity (fifth bar, $p<0.001$). As expected, in the absence of AhREs in the reporter construct, TCDD had no further effect on reporter activity (sixth bar). These results suggest that hyper-activation of the AhR with environmental chemicals in Hs578T cells has no effect on *c-myc* transcription.

4. Inhibition of AhR activity de-repressess c-myc promoter activity.

Experiments with *pGL3-c-myc* mutants are consistent with a role for the AhR in constitutively repressing *c-myc* transactivation. However, the formal possibility that deletion of NF-κB binding sites and mutation of all six AhREs resulted in changes that could affect transcription factors other than NF-κB and AhR could not be ruled out. Therefore, a second approach to evaluating putative AhR repression of *c-myc* transcription was taken. An evolutionarily conserved AhR repressor (AhRR) has been described in several species. The AhRR potently inhibits AhR-dependent *CYP1A1* activity by competing for the AhR binding partner ARNT and by blocking AhR-AhRE binding. AhRR-ARNT complexes are transcriptionally inactive. Notably, AhRR derived from killifish (*F. heteroclitus*) inhibits both human and mouse AhR-dependent transactivation in an AhR-specific manner. In preliminary experiments, the *F. heteroclitus* AhRR (*FhAhRR*) was more effective at suppressing *pGudLuc* activity in Hs578T cells than a human *AhRR* expression construct. Therefore, the *FhAhRR* construct was chosen to test the prediction that inhibition of constitutive AhR activity in Hs578T cells would de-repress AhR-dependent *c-myc* promoter transactivation.

Figure 6

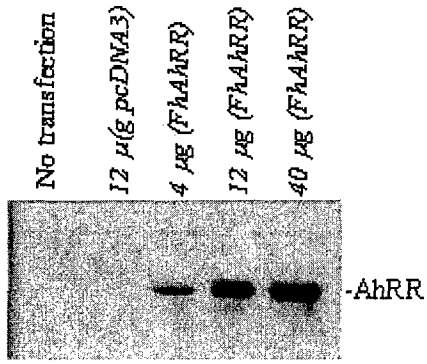

Figure 6 AhR repressor (AhRR) expression after Hs578T cell transfection. *Hs578T cells were transfected with 0.5 μg of pcDNA3.1 or pcDNA3-FhAhRR (FhAhRR). Twenty four hours later cells were harvested, lysed, and 12-40 μg of protein loaded into SDS-PAGE gels for western immunoblotting with a purified species-specific AhRR-specific antibody. Immunoblotting of stripped gels with β-actin-specific antibody confirmed relative levels of protein loaded (not shown). Representative data from a total of three experiments are presented.*

Figure 7A

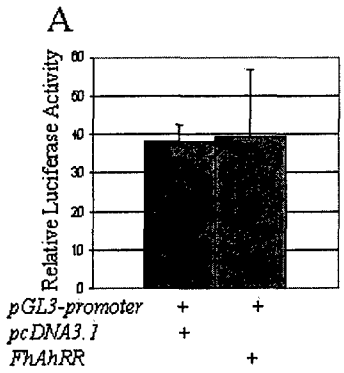

Figure 7B

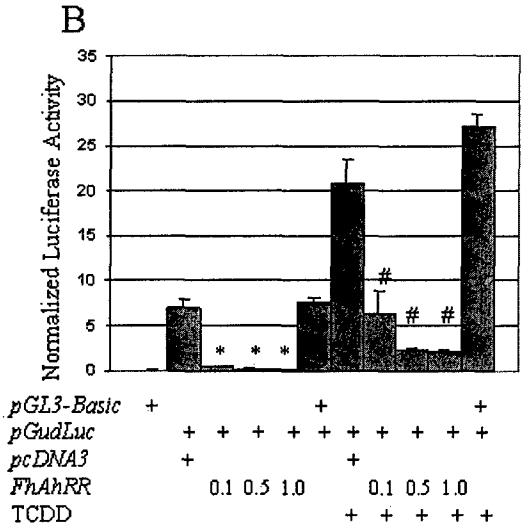

Figure 7C

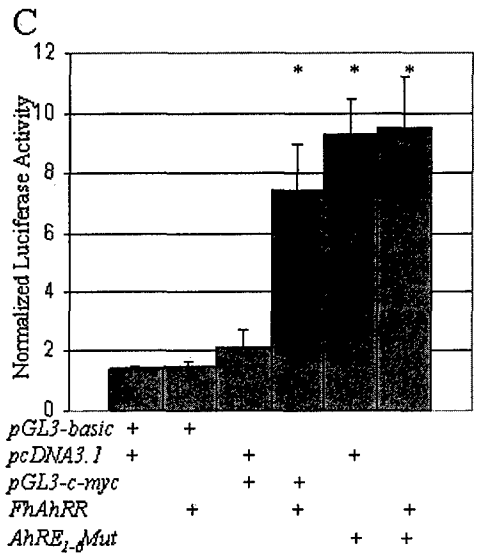

Figure 7 AhR repressor specifically inhibits constitutive CYP1A1 and induces pGL3-c-myc promoter activity. *A) Hs578T cells were co-transfected with 1.0 μg of a firefly luciferase reporter construct driven by the SV40 promoter (pGL3-promoter), 0.5 μg control vector (pcDNA3.1) or 0.5 μg pcDNA3-FhAhRR (FhAhRR). Cells were harvested twenty four hours later and assayed for reporter activity as above. Data pooled from five experiments are presented as the average normalized firefly luciferase activity ± standard error.* ***B)*** *Hs578T cells were co-transfected in triplicate wells with control vectors pGL3-basic or pcDNA3.1, with 0.1 μg pGudLuc and 0.05 μG phRL-TK, and with titered concentrations of pcDNA3-FhAhRR (FhAhRR) as indicated. Twenty four hours later cultures were treated with 10^{-9} M TCDD where indicated. Cells were harvested twenty four hours later and lysates assayed for firefly and renilla luciferase activity. Data pooled from three experiments are presented as the average normalized firefly luciferase activity ± standard error. An asterisk (*) indicates a significant decrease in normalized pGudLuc reporter activity in FhAhRR-transfeced cells relative to cells transfected with the control vector, pcDNA3.1, $p<0.001$. A pound sign (#) indicates a significant decrease in normalized pGudLuc reporter activity in TCDD-treated, FhAhRR-transfected cells relative to cells transfected with control vector, pcDNA3.1, and treated with TCDD, $p<0.008$.* ***C)*** *Hs578T cells were transfected with1.0 μg pGL3-basic, wildtype pGL3-c-myc, or AhRE mutant (pGL3-AhRE$_{1-6}$Mut) reporter plasmids, 0.05 μg phRL-TK, and, where indicated, 1.0 μg pcDNA3-FhAhRR (FhAhRR) or control plasmid pcDNA3.1. Cells were harvested twenty four hours later and lysates assayed for firefly and renilla luciferase activity. Data pooled from five experiments are presented as the average normalized firefly luciferase activity ± standard error. An asterisk indicates a significant increase in normalized reporter activity relative to cells transfected with pGL3-c-myc and control vector pcDNA3.1, $p<0.007$.*

Significant levels of FhAhRR were detected in Hs578T cells 24 hours after transfection with *FhAhRR* (Figure 6). As expected from previous experiments demonstrating the specificity of the AhRR (Karchner et al., 2002), *FhAhRR* transfection had no effect on SV40 promoter-driven, *pGL3-promoter* reporter activity (Figure 7A). As in previous experiments, transfection of the *CYP1A1* promoter-driven *pGudLuc* reporter plasmid resulted in a significant (~7-fold) increase in luciferase activity (Figure 7B, second bar) that was further augmented by addition of TCDD (seventh bar). Co-transfection of cells with *pGudLuc* and 0.1, 0.5, or 1.0 μg *FhAhRR* ablated the background levels of reporter activity (bars 3-5, $p<0.001$) and significantly reduced the TCDD-induced reporter activity (bars 8-10, $p<0.008$). These data confirm that *FhAhR*R is a potent inhibitor of both the constitutive and inducible human AhR in these tumor cells.

As in previous experiments, transfection of Hs578T cells with the wildtype *pGL3-c-myc* reporter resulted in a low level of reporter activity which, in this series of experiments, was not significantly greater than the background activity in *pGL-basic*-transfected cells (Figure 7C, third bar). Importantly, co-transfection of *pGL3-c-myc* with 1.0 μg *FhAhRR* significantly ($p<0.007$) increased reporter activity 3-4 fold (fourth bar). As expected, transfection with a construct in which all AhREs were mutated increased reporter activity (fifth bar, $p<0.007$) and co-transfection with the *FhAhRR* had no further effect on the *pGL3-AhRE$_{1-6}$Mut* luciferase activity (sixth bar). These data are consistent with the conclusion that the AhR constitutively represses *c-myc* transactivation through its interaction with at least some of the AhREs.

5. AhR-mediated repression of endogenous c-myc

While the data described above support regulation of tumor cell *c-myc* transcription by the AhR, they do not address the potential regulation of steady state *c-myc* levels by the AhR *in situ*. To determine if constitutively active AhR affects the steady state levels of *c-myc*, Hs578T cells were transfected with *FhAhRR* or control vector (*pcDNA3.1*) and *c-myc* mRNA levels quantified 24 hours later by real-time PCR. Indeed, *c-myc* mRNA levels almost doubled following transfection with *FhAhRR* (Figure 8, $p<0.05$). Although many factors contribute to maintenance of steady state mRNA levels, these data suggest that at least one contributor to the down-regulation of *c-myc* mRNA is constitutively active AhR.

Figure 8

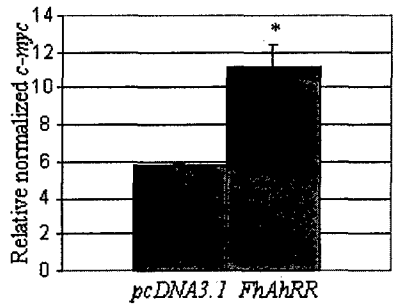

Figure 8 AhR repressor increases endogenous c-myc mRNA. *Hs578T cells were transfected with 0.5 μg control vector pcDNA3.1 or with 0.5 μg pcDNA3-FhAhRR (FhAhRR). Twenty four hours later cells were harvested, RNA extracted, and c-myc RNA quantitated by real-time PCR. Data are pooled from three experiments and are presented as the average normalized c-myc levels ± standard error. An asterisk (*) indicates a significant increase in c-myc mRNA, $p<0.05$.*

Key Research Accomplishments

1. Our results indicate that the AhR constitutively binds the *c-myc* promoter;
2. there is a low but significant baseline level of *c-myc* promoter activity which is not regulated by NF-κB and is not affected by an environmental AhR ligand;
3. deletion of any one of the AhREs has no effect on constitutive reporter activity while deletion of all six increases reporter activity approximately five fold;
4. A similar increase in reporter activity occurs when constitutively active AhR is suppressed by transfection with an AhR repressor plasmid (*AhRR)*;
5. *AhRR* transfection significantly increases background levels of endogenous *c-myc*.
6. These results suggest that the AhR represses *c-myc* and that AhR up-regulation in transformed cells may represent an attempt to regulate cell growth or death through AhR-*c-myc* interactions.

Reportable Outcomes

Yang, X., Murray, T.J., Liu, D., and Sherr D.H.
AHR Regulation of c-Myc in human breast cancers.
-Oral presentation, 43rd Annual Meeting of Society of Toxicology, Baltimore, MD, 2004.

Abstract Number: 643 **Day / Time:** Tuesday, Mar. 23, 10:10 AM - 10:30 AM

AHR REGULATION OF C - MYC IN HUMAN BREAST CANCERS

X.Yang; T.J.Murray; D.Liu; D.H.Sherr. Enviormental Health, Boston University School of Public Health, Boston, MA.

It has long been suggested that ubiquitous environmental chemicals, such as PAH, contribute to human breast cancer. The preferential targeting of breast tissue by orally administered PAH in rodent breast cancer models supports this contention. Most of the biologic activity of PAH and related dioxins is mediated by the AhR. Previously, we demonstrated that the AhR and CYP1B1, an AhR-regulated gene, are dramatically up-regulated in rodent and human breast tumors. Here, we investigated the possible consequences of this apparent constitutive AhR activation. In specific, we tested if constitutively active AhR in a human breast cancer cell line, regulates c-myc, an important breast cancer oncogene which contains six AhR binding sites (AhREs) in its promoter. Luciferase reporter vectors containing the c-myc promoter with its six AhREs was constructed. Variants with mutations in NF-kB and/or AhR binding sites were generated by site-directed mutagenesis. These constructs were transfected into Hs578T cells and subsequently assayed for luciferase activity in the presence or absence of TCDD, a strong AhR agonist. The results indicate that: 1) there is a significant baseline level of wildtype c-myc promoter driven reporter activity in these tumor cells which was not affected by inclusion of TCDD, 2) the baseline reporter activity was not affected by deletion of the NF-kB site, 3) while mutation of single AhRE sites had no effect on baseline reporter activity, mutation of all six sites resulted in a five fold increase in reporter activity; a similar increase in reporter activity was seen when the wildtype reporter construct was co-transfected with an AhR repressor plasmid, 4) c-myc-specific real time PCR indicated that AhR repressor transfection increased background levels of endogenous c-myc mRNA. These results suggest that the AhR represses c-myc transcription and that AhR up-regulation in tumor cells may represent a failed growth feedback mechanism.

Citation: X.Yang, T.J.Murray, D.Liu, D.H.Sherr. AHR REGULATION OF C - MYC IN HUMAN BREAST CANCERS. Abstract No. 643. *2004 Itinerary Planner.* Baltimore, MD: Society of Toxicology